Papahadjopoulos, D., & Hanahan, D. J. (1964) *Biochim. Biophys. Acta 90*, 436.
Papahadjopoulos, D., Vail, W. J., Pangborn, W. A., & Poste, G. (1976) *Biochim. Biophys. Acta 448*, 265.
Subbiah, P. V., Bajwa, S. S., Smith, C. M., & Hanahan, D. J. (1976) *Biochim. Biophys. Acta 444*, 131.
Suttie, J. W., & Jackson, C. M. (1977) *Physiol. Rev. 57*, 1.
Suurkuusk, J., Lentz, B. R., Barenholz, Y., Biltonen, R. L., & Thompson, T. E. (1976) *Biochemistry 15*, 1393.
Tracy, P. B., Peterson, J. M., Nesheim, M. E., McDuffie, F. C., & Mann, K. G. (1979) *Thromb. Haemostasis* (in press).
Weber, K., & Osborn, M. (1969) *J. Biol. Chem. 244*, 4406.

Interaction of Divalent Cations and Polymyxin B with Lipopolysaccharide†

Melvin Schindler and M. J. Osborn*

ABSTRACT: Cation binding to the lipopolysaccharide of *Salmonella typhimurium* was investigated by equilibrium dialysis and fluorometric titration of lipopolysaccharides derivatized with dimethylaminonaphthalenesulfonyl chloride (dansyl chloride). In the presence of Tris buffer (50 mM), addition of Ca^{2+} or Mg^{2+} resulted in a blue shift in the emission maximum and an increase in the relative fluorescence of dansylated lipopolysaccharides obtained from the rough mutants G30 (galactose deficient, R_c chemotype) and G30A (heptose deficient, R_e chemotype). These effects were completely reversed by EDTA. Fluorometric titrations revealed two types of binding sites with markedly different affinities for divalent cations in both lipopolysaccharides. The first class, of relatively low affinity, gave K_d values of 0.2 and 1–2 mM with G30A and G30, respectively, and was attributed to pyrophosphoryl and/or phosphodiester groups of the 3-deoxy-*manno*-octulosonate (KDO)–lipid A region of the molecule. The second class, of higher affinity, yielded K_d values for Ca^{2+} and Mg^{2+} of 6 and 15 μM, respectively, with both lipopolysaccharides. Titrations with polymyxin B showed only high-affinity binding with K_d values of 0.3 μM for G30A and 0.5 μM for G30. The nature of the high-affinity Ca^{2+} and Mg^{2+} binding site was further investigated by equilibrium dialysis of lipopolysaccharides and related products with $^{45}Ca^{2+}$. All lipopolysaccharides tested (wild type, G30, and G30A) bound 1 mol of Ca^{2+} per mol of lipopolysaccharide monomer with a K_d of 12–13 μM. In contrast, lipid A (obtained by mild acid hydrolysis of lipopolysaccharide) and an incomplete biosynthetic precursor of lipid A lacking 3-deoxy-D-*manno*-octulosonate yielded K_d values of 100 and 56 μM and a reduction in the stoichiometry of binding to 0.5 and 0.3 mol of Ca^{2+} per mol, respectively. The results suggest that the branched 3-deoxy-D-*manno*-octulosonate trisaccharide unit of lipopolysaccharide may afford a specific high-affinity site for interaction with divalent cations required for assembly and maintenance of the normal structural organization of the outer membrane.

It now appears well established that the lipopolysaccharides located in the external leaflet of the outer membrane of gram-negative enteric bacteria and their interactions with cations are important for the barrier function of the membrane (Leive, 1974; Sanderson et al., 1974; Galanos et al., 1977). Lipopolysaccharide contains a number of potential cation binding sites, and electrodialysis is necessary to remove tightly bound inorganic cations and polyamines from isolated lipopolysaccharide (Galanos & Luderitz, 1975). Both lipid A and the backbone region of the polysaccharide contain phosphate, and in addition a cluster of three carboxyl groups is afforded by the unique branched KDO[1] trisaccharide unit which links polysaccharide to lipid A (Figure 1). It has been postulated by Leive (1974) and others (van Alphen et al., 1978) that divalent cations form ionic bridges between neighboring lipopolysaccharide phosphate groups, stabilizing the outer-membrane structure. However, it is also possible that the KDO residues can form a cage to coordinate Ca^{2+}, Mg^{2+}, or other cations, as has been established for sialic acid. Jaques et al. (1977) have measured 1:1 associations of Ca^{2+} to sialic acid, while Sillerud et al. (1978) have found that the single sialic acid in GM_1 ganglioside was intimately involved through its carboxyl and glycero side chain with cation binding. In fact, other sugars linked to sialic acid also contributed to the metal cation coordination sphere, thus raising the affinity constant beyond that of sialic acid alone.

To define more clearly the nature of the metal binding sites of lipopolysaccharide, we have employed fluorescence analysis of dansylated derivatives of various lipopolysaccharides. In addition, equilibrium dialysis measurements were performed with $^{45}Ca^{2+}$ to provide information about the stoichiometry of binding. The results indicate that the lipopolysaccharides contain a high-affinity binding site for Ca^{2+} and Mg^{2+} which appears to be formed by the three KDO residues of the molecule.

Materials and Methods

Materials

Bacterial Strains and Media. Salmonella typhimurium LT2 and strain G30, a mutant of *S. typhimurium* LT2 lacking UDPgalactose-4-epimerase, have been described (Osborn et al., 1962). Strain G30A is a mutant of G30 which produces a heptoseless lipopolysaccharide (Osborn et al., 1972).

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[1] Abbreviations used: KDO, 3-deoxy-D-*manno*-octulosonic acid; DNS, dimethylaminonaphthalenesulfonyl chloride; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; AraN, 4-amino-4-deoxy-L-arabinose.

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Table I: Composition of Lipopolysaccharide Preparations

component	molar equiv		
	LT2	G30	G30A
glucosamine	3.00	2.00	2.00
phosphate	5.50	5.19	2.46
KDO[a]		1.45	1.30
fatty acids			
12:0 + 14:0	4.75	2.32	2.13
14:OH	4.13	4.60	2.03
other	3.63	1.37	1.39
total	12.51	8.29	5.55
amino acids[b]	≤0.04	≤0.04	≤0.04

[a] Values obtained by the thiobarbituric acid method; the heptose-linked residue of KDO is unreactive in this assay (Dröge et al., 1970). The value for LT2 was not determined because of interference by abequose. [b] Moles of lysine per mole of lipopolysaccharide. Lysine was the major peak on all amino acid analyses.

Cultures were grown at 37 °C with vigorous aeration in proteose peptone–beef extract medium (PPBE) (Osborn et al., 1962).

Chemicals. All radiochemicals and Liquiflor were purchased from New England Nuclear. Bio-Solv BBS-3 was purchased from Beckman Instruments. DNS and polymyxin B were obtained from Sigma Chemical Co., and Schardinger-β-Dextrin (cyclodextrin) was from Pierce. Fluorescamine is a product of Hoffmann-La Roche. All other chemicals used were of the highest purity commercially available.

Lipopolysaccharides. These were isolated by aqueous phenol extraction (Romeo et al., 1970) or by the phenol–chloroform–petroleum ether method (Galanos et al., 1969). Residual contaminating phospholipid was removed from LT2 and G30 lipopolysaccharides by extraction with $CHCl_3$–methanol (2:1) and from G30A by filtration on a column of Sephadex LH-20 (sample applied in the $CHCl_3$–methanol solution). Elution was with $CHCl_3$–methanol (2:1). To remove cationic contaminants, all lipopolysaccharide preparations were electrodialyzed by the procedure of Galanos & Luderitz (1975), neutralized with triethylamine, dialyzed against water, and then lyophilized. Stock solutions were prepared by dissolving a weighed amount of lipopolysaccharide in water and sonicating in a Megason ultrasonic cleaner until the solution was optically clear. The composition of the purified lipopolysaccharides is summarized in Table I.

^{14}C-Labeled G30 lipopolysaccharide was prepared by growing G30 in the presence of [^{14}C]galactose (Osborn et al., 1962) while ^{14}C-labeled G30A was obtained by growth in *N*-acetyl-D-[^{14}C]glucosamine (Rick et al., 1977). The lipid A precursor was prepared as described by Rick et al. (1977). Lipid A was obtained by hydrolysis of G30 lipopolysaccharide in 0.1 N acetic acid for 1 h at 100 °C as described (Osborn et al., 1972).

Methods

Colorimetric Assays. Glucosamine was determined by the Elson–Morgan reaction as modified by Strominger et al. (1959). Organic and inorganic phosphate were determined as described by Ames (1966). KDO was determined by the thiobarbituric acid assay (Osborn, 1963).

Gas Chromatography. Fatty acids were analyzed as their methyl esters. Preparation and analysis were performed as described in Rick et al. (1977).

Amino Acid Analysis. All lipopolysaccharides were subjected to amino acid analysis in the following manner. Approximately 0.6–1.5 μmol of lipopolysaccharide was treated with 6 N HCl (0.5 mL) at 110 °C in vacuo for 20 h. The HCl was removed in vacuo over NaOH pellets, and the residue was dissolved in sodium citrate buffer (0.2 M Na^+), pH 2.2. Norleucine was added to serve as an internal standard for comparison. The sample was analyzed on the long and short columns of a Beckman-Spinco amino acid analyzer.

Amino sugar analysis was performed on the long column of the amino acid analyzer. Lipopolysaccharide (30–60 nmol of glucosamine) was hydrolyzed in 4 N HCl (0.5 mL) for 18 h at 100 °C in a Teflon-lined screw-top tube. HCl was removed in vacuo over NaOH pellets, and the sample was dissolved in sodium citrate buffer. A color constant for glucosamine was determined by hydrolysis of pure *N*-acetyl-D-glucosamine in parallel with the sample.

Fluorescamine Assay. Free amino groups were determined by reaction of fluorescamine with primary amines to give a fluorescent species (Weigele et al., 1972). Lipopolysaccharide and the products of mild acid hydrolysis were reacted with fluorescamine and compared with ribonuclease and glycine standards. The reaction buffer was 0.2 M $NaHCO_3$, pH 8.5. Analysis was performed within 1 h of reaction on a Hitachi Perkin-Elmer MPF-3 spectrofluorometer using quartz microcells.

Dansylation of Lipopolysaccharide. DNS was prepared as an included compound of Schardinger-β-Dextrin (cyclodextrin) as described (Kinoshita et al., 1974). Each lipopolysaccharide (2 mg/mL) was incubated with 10–15 mg of DNS–cyclodextrin complex in 0.2 M $NaHCO_3$, pH 8.5, for 1 h with constant shaking at room temperature and centrifuged at 1000 rpm for 5 min to sediment the cyclodextrin. The supernatant fraction was then chromatographed on a Sephadex G-25 column (1.5 × 20 cm), employing water as the eluant. Derivatized lipopolysaccharide was eluted as a single peak in the void volume and was lyophilized and stored at 4 °C until use.

Fluorescence Measurements. All fluorescence titrations and spectra (uncorrected) were performed on a Hitachi Perkin-Elmer MPF-3 spectrofluorometer. Association measurements were carrried out with an excitation wavelength of 340 nm and emission at the wavelength of maximal spectral change for the complex (460 nm for DNS–G30A and 480 nm for DNS–LT2 and DNS–G30) in buffers containing 50 mM Tris-HCl, pH 7.5. Tris titrations were performed in 10 mM Hepes, pH 7.5. The relative fluorescence, *F*, of the DNS–lipopolysaccharide in the presence of cation was determined by comparison of two cuvettes containing identical concentrations (1–3 μM of lipopolysaccharide, $A_{280} \ll 0.1$ in a 1-cm cell) in the presence and absence of cation. The concentration of stock cation solutions was determined by dry weight of the appropriate compound. In the case of lipopolysaccharide, amino sugar determination on the amino acid analyzer was found to be the most accurate method of quantitation.

The dissociation constants for the complexes were calculated by a nonlinear least-squares fit of the data as defined by Schindler et al. (1977).

Equilibrium Dialysis. Experiments were performed with a Hoefer Scientific Instruments minidialyzer. A prototypic experiment involved adding 0.2 mL of lipopolysaccharide and $^{45}Ca^{2+}$ solution (13.8 mCi/mg) to one side of each cell and 0.2 mL of buffer to the other. In all measurements, the buffer chosen was 100 mM Tris, pH 7.5, which proved adequate in preliminary experiments to minimize surface charge effects caused by possible lipopolysaccharide aggregates. With all cells filled, the cell block was rotated at about 0.2 rotations/s at room temperature for the desired time, usually 15 h. Mixing action was provided by rotation and the movement of a remaining air bubble in each chamber. The cells were sealed

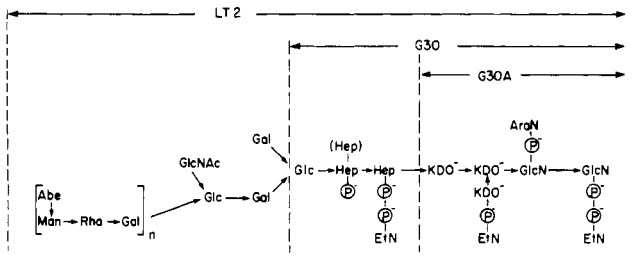

FIGURE 1: Structure of the lipopolysaccharide of *S. typhimurium*. Fatty acids linked to the diglucosamine backbone have been omitted. Abbreviations used: Hep, L-glycero-D-*manno*-heptose; Abe, abequose; Rha, L-rhamnose; EtN, ethanolamine; AraN, 4-amino-4-deoxy-L-arabinose.

Table II: Determination of Amino Groups in Lipopolysaccharides[a]

lipopoly-saccharide	amino groups (mol/mol of lipopolysaccharide) total	lipid A[b]	poly-saccharide[b]	AraN[c]
G30A	1.13	0.14	0.27	0.72
G30	1.18	0.13	0.61	0.44
LT2	0.80	0.33	0.29	0.18

[a] Determined by fluorescamine assay as described under Methods. [b] Lipid A was obtained by mild acid hydrolysis as described under Methods. Lipid A was recovered by centrifugation (20 000 rpm; 5 min) and washed. The supernatants after hydrolysis contained the polysaccharide components. These were then lyophilized and assayed. [c] The AraN content was derived by subtracting the amino-group content of lipid A and polysaccharide from the total amount of amino groups in intact lipopolysaccharide.

with a strip of transparent tape.

Dialysis membranes were purchased from Hoefer and were prepared by boiling with EDTA–sodium bicarbonate for 10 min, followed by extensive washing with distilled water. Membranes were stored in cold 50% ethanol.

$^{45}Ca^{2+}$ equilibrated across the membrane in 10 h in the absence of lipopolysaccharide. No diffusion of ^{14}C-labeled G30A or G30 lipopolysaccharide could be detected through the membrane in the time course of the experiments. A typical dialysis experiment consisted of eight runs at different $^{45}Ca^{2+}$ concentrations. The lipopolysaccharide concentration was 5–10 μM based on monomer molecular weight. After equilibration, three 50-μL aliquots were taken from each side of each cell for scintillation counting. Scatchard plots (Scatchard, 1949) were employed to analyze the data. Dialysis measurements performed for 1 and 2 days gave the same results.

Counting Procedures. Solutions were counted in toluene–Liquiflor–Biosolv BBS-3 (12.5%). The samples were made up to 0.5 mL with H_2O, and 5 mL of scintillation fluid was added. Radioactivity was measured in a Beckman LS-230 liquid scintillation counter.

Results

Characterization of DNS-Labeled Lipopolysaccharides. The postulated structures of the lipopolysaccharides of wild-type *S. typhimurium* LT-2 and the mutants G30 and G30A are summarized in Figure 1. Both lipid A and the saccharide chains contain free amino groups as ethanolamine and 4-amino-4-deoxy-L-arabinose residues in close proximity to potential cation binding constituents. Amino group analyses of the isolated lipopolysaccharides (Table II) revealed considerable microheterogeneity in amino group content and distribution as shown by substoichiometric values, as well as significant differences between the three lipopolysaccharide types. These findings are in agreement with the previous results of Lehmann et al. (1978), and the values obtained for G30 lipopolysaccharide are very similar to the figures reported by these workers for another *galE* mutant, SL761. In all cases, however, analysis of the products of mild acid hydrolysis confirmed the presence of derivatizable amino groups in both the saccharide and lipid A portions of the molecule (Table II).

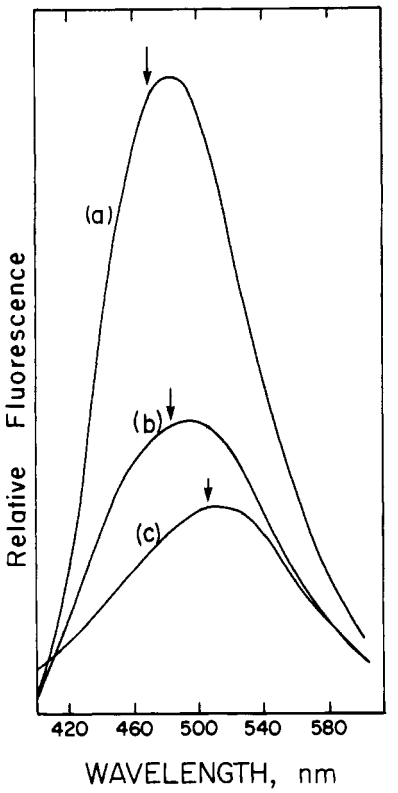

FIGURE 2: Fluorescence emission spectra of DNS–lipopolysaccharides at pH 7.5 in 50 mM Tris-HCl buffer. (a) DNS–G30A; (b) DNS–G30; (c) DNS–LT2. The arrows refer to the emission maxima in 10 mM Hepes, pH 7.5.

Emission spectra of the DNS–lipopolysaccharides in Tris buffer are shown in Figure 2. Spectra of the lipid A and saccharide fractions obtained by mild acid hydrolysis showed that dansyl groups were incorporated into both portions of all three lipopolysaccharides; representative emission spectra of the isolated lipid A and KDO fractions of DNS–G30A are illustrated in Figure 3. As might be expected, the emission spectrum of lipid A (λ_{max} = 470–490 nm) indicated a relatively hydrophobic probe environment, while that of the lipid-free saccharide (λ_{max} = 550–560 nm) was hydrophilic. Spectra of the intact lipopolysaccharides resembled those of the isolated lipid A's. This presumably reflected the preponderance of amino groups in the lipid, as well as a probable decrease in hydrophilicity of saccharide-linked chromophores in the intact molecule.

Quantitation of Cation–Lipopolysaccharide Dissociation Values. Addition of 5 mM Ca^{2+} to G30 DNS–lipopolysaccharide in 50 mM Tris, pH 7.5, caused an enhancement and blue shift of the fluorescence emission maximum (Figure 4). This was abolished by addition of 10 mM EDTA (10 mM EDTA alone had no effect). An enhancement and a shift of similar magnitude were obtained with DNS–lipopolysaccharide from G30A; a small effect was also observed with the wild-type LT2 derivative but was not large enough to permit accurate titrations. Titration of G30 and G30A DNS–lipopolysaccharides with Ca^{2+} revealed two saturable sites which differed widely in their apparent affinities for Ca^{2+}. A

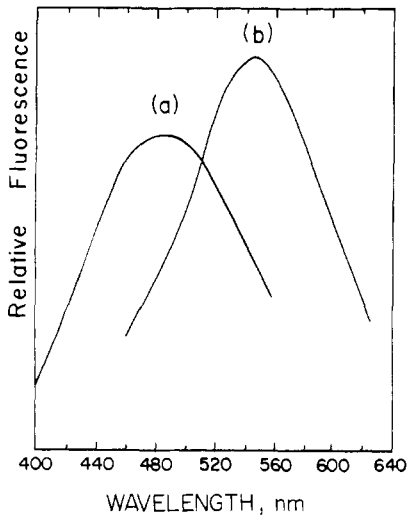

FIGURE 3: Emission spectra of DNS–lipid A and –polysaccharide components of acid-hydrolyzed DNS–G30A. Dansylated G30A (1 mg) was hydrolyzed, and the reaction products were separated as described in the legend to Table II. The buffer used was 50 mM Tris, pH 7.5, and the excitation wavelength was 340 nm in all cases. (a) DNS–lipid A (0.04 mg/mL); (b) DNS–polysaccharide (0.05 mg/mL).

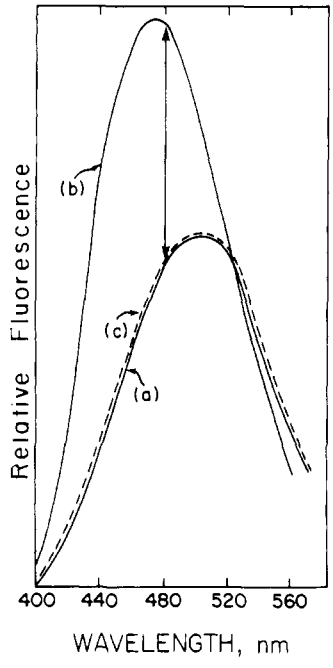

FIGURE 4: Effect of Ca^{2+} on the emission spectrum of DNS–G30 lipopolysaccharide in 50 mM Tris, pH 7.5. (a) DNS–G30; (b) DNS–G30 + 5 mM Ca^{2+}; (c) (b) + 10 mM EDTA. The arrow indicates fluorescence enhancement. Excitation was at 340 nm.

representative titration curve for G30 is shown in Figure 5. Mg^{2+} yielded fluorescence changes similar to those with Ca^{2+}, and titration curves also showed high- and low-affinity binding sites in the same concentration ranges. Dissociation constants were calculated by employing a nonlinear least-squares fit program (Schindler et al., 1977). K_d values so obtained for Ca^{2+}, Mg^{2+}, and the cationic antibiotic, polymyxin B, are summarized in Table III. Typically, the uncertainty in the calculated K_d from a single titration was less than ± 0.2 μM for the high-affinity site. The repetition of a titration (whether at the same or a different emission wavelength) gave results reproducible within the same uncertainty.

The observed changes in fluorescence were dependent upon the presence of Tris. When DNS–lipopolysaccharide was titrated in 10 mM Hepes, no dramatic changes such as those shown in Figure 4 were obtained. Comparison of emission spectra in 50 mM Tris and 10 mM Hepes showed that Tris quenched the DNS fluorescence and shifted the emission maximum to longer wavelengths. This effect was reversed on addition of Ca^{2+}, Mg^{2+}, or polymyxin B. The effect of Tris

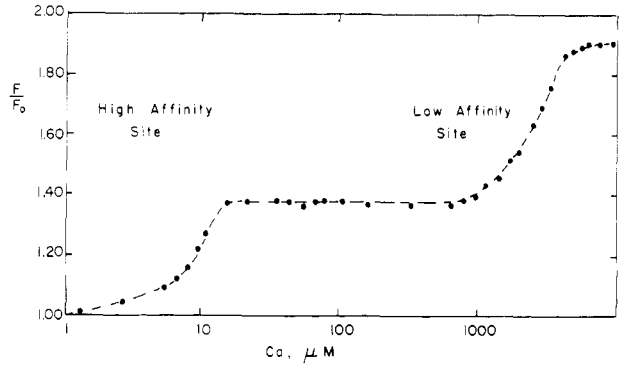

FIGURE 5: Fluorescence titration of DNS–G30 with $CaCl_2$ in 50 mM Tris, pH 7.5, at an emission wavelength of 480 nm. Excitation wavelength was set at 340 nm.

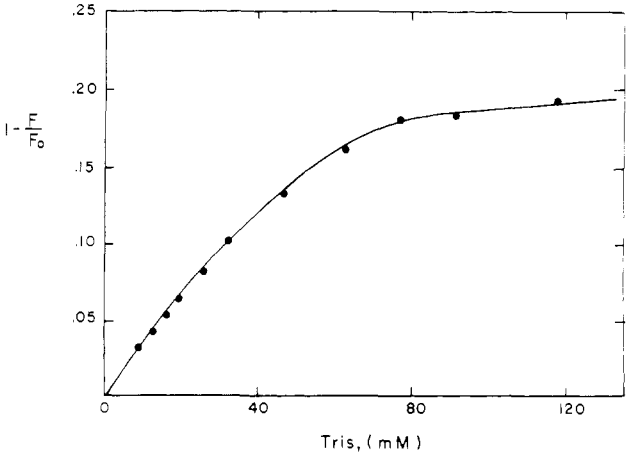

FIGURE 6: Fluorescence titration of Tris binding to DNS–G30A and DNS–G30. Titrations were performed at an emission setting of 510 nm.

Table III: Dissociation Constants (K_d) of Cations to Lipopolysaccharides As Determined by Fluorescence Titration and Equilibrium Dialysis

lipopolysaccharide	fluorescence titration[a] high-affinity site (μM)	fluorescence titration[a] low-affinity site (mM)	equilibrium dialysis,[a] high-affinity site (μM)
lipid A precursor + Ca^{2+}	*b*		56
lipid A + Ca^{2+}	*b*		100
G30A + Ca^{2+}	6.0	0.2	12.5
G30A + Mg^{2+}	14.0	0.2	
G30A + polymyxin	0.3[c]		
G30 + Ca^{2+}	6.0	2	12.0
G30 + Mg^{2+}	15.0	1	
G30 + polymyxin	0.5[c]		
LT2 + Ca^{2+}	*d*		13.0

[a] Described under Methods. The buffer used for fluorescence titration was 50 mM Tris-HCl, pH 7.5, while, for equilibrium dialysis, 100 mM Tris-HCl, pH 7.5, was employed. [b] Lipid A precursor has no amino groups for derivatization. [c] Possibly two binding sites per diglucosamine unit of lipopolysaccharide. [d] Fluorescence changes were not sufficient for accurate titration.

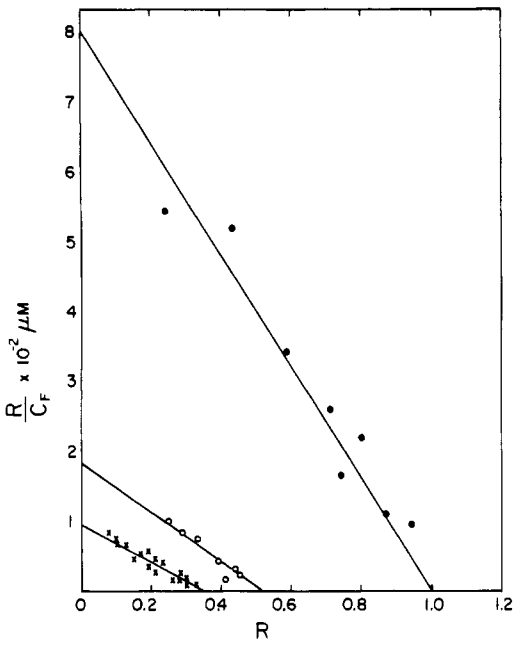

FIGURE 7: Scatchard analysis of $^{45}Ca^{2+}$ binding to G30 lipopolysaccharide, lipid A, and lipid A precursor as measured by equilibrium dialysis. G30 lipopolysaccharide (●); precursor (○); lipid A (×).

was concentration dependent, and titration of both DNS–G30A and DNS–G30 with Tris HCl in 10 mM Hepes buffer showed saturable binding with a K_d of 35 mM (Figure 6).

Equilibrium Dialysis Studies. In order to characterize further the high-affinity Ca^{2+} and Mg^{2+} site and evaluate the stoichiometry of cation binding to this site, we employed equilibrium dialysis to measure $^{45}Ca^{2+}$ binding to lipopolysaccharides, isolated lipid A, and a KDO-deficient biosynthetic precursor of lipid A (Rick et al., 1977). Figure 7 offers a compilation of these results in a Scatchard plot (Scatchard, 1949), and K_d values determined by this method are listed in Table III. The stoichiometry of binding for G30A, G30, and LT2 lipopolysaccharides was one Ca^{2+} per monomeric lipopolysaccharide chain, assuming two GlcN residues per chain for G30 and G30A and three for the LT2 wild type (cf. Figure 1). K_d values were essentially identical for all three lipopolysaccharides and were comparable to those derived by fluorescence titration of DNS derivatives. Both lipid A and the lipid A precursor (which lacks the ester-linked saturated acyl chains of lipid A as well as KDO) showed a reduction in the stoichiometry of Ca^{2+} binding to approximately 0.5:1 and 0.3:1, respectively, and a marked increase in apparent K_d.

Discussion

A variety of cations are known to bind tenaciously to lipopolysaccharide (Galanos & Lüderitz, 1975), and the possible role of lipopolysaccharide–cation interactions in outer-membrane assembly (Galanos et al., 1977; Verkleij et al., 1977; van Alphen et al., 1978) and stabilization (Leive, 1974) has received considerable attention. To our knowledge, however, characterization of cation binding sites has not previously been reported. Dansylation of free amino groups of lipopolysaccharide provided a convenient tool to monitor binding. Amino groups are found in both the polysaccharide backbone and the lipid A region of the molecule, and, although these appear to be present in substoichiometric amounts [Table II and Lehmann et al. (1978)], attached DNS groups function effectively as reporters for cation interactions. Dansyl groups were incorporated into both the lipid A and polysaccharide components, and it is not known which of the several possible sites of dansylation were responsive to addition of cations. However, the similarity in response of DNS–G30 and G30A lipopolysaccharide suggests that probe residues in the KDO–lipid A region were primarily responsible for the observed fluorescence changes.

Upon addition of Ca^{2+}, Mg^{2+}, or polymyxin B to a Tris-buffered solution of dansylated G30 and G30A lipopolysaccharide, a large blue shift was observed in the emission spectrum with an increase in relative fluorescence. K_d values determined from the fluorescence changes showed that in DNS–G30A and DNS–G30 there are at least two sites of coordination for Ca^{2+} and Mg^{2+} with widely differing affinities. Since G30A lipopolysaccharide lacks the heptose-linked phosphorylethanolamine (Figure 1), it would appear that this group can be ruled out as either the high- or low-affinity site, and the KDO carboxylates and glucosamine phosphates then remain as the most likely candidates for a high-affinity site. This conclusion was also reached by Bader & Teuber (1973) with regard to a polymyxin binding site. To differentiate between these two possibilities, we employed equilibrium dialysis of G30, G30A, and wild-type lipopolysaccharides, lipid A, and a biosynthetic precursor of lipid A lacking KDO (Rick et al., 1977). All lipopolysaccharides, which contain the three KDO groups, showed a stoichiometry of 1 mol of Ca^{2+} per mol of lipopolysaccharide, while those derivatives without KDO (lipid A and the lipid A precursor) yielded a stoichiometry of 0.5 or less and a 5- to 10-fold reduction in K_d. It thus appears that the three KDO sugars affect a large change in cation complexation as witnessed both by stoichiometry and by dissociation constants.

The observed effects of Tris ion on the fluorescence properties of DNS–lipopolysaccharides and their response to divalent cations indicate that Tris binds competitively with these ions to both the high- and low-affinity sites. Fluorescence titrations yielded an apparent K_d for Tris of 35 mM but did not distinguish between the two classes of metal binding sites. These results are of interest in light of the known effects of Tris on the structural organization of the outer membrane. Removal of lipopolysaccharide from the outer membrane of gram-negative bacteria by EDTA is markedly enhanced by Tris buffer (Leive, 1974; Voss, 1967), and the synergistic effect is relatively specific for Tris ion. The observation of Voss (1967) that tris(hydroxymethyl)nitromethane is inactive indicated that the free amino group of Tris is essential for its disruptive effect. This is consistent with our findings that the amino function of Tris competes with other cations for specific binding to both sites in the molecules.

Mono-, di-, and triphosphates have been shown to bind metals and polyamines in the millimolar range (Williams, 1972). Binding of Ca^{2+} to phosphatidic acid (Abramson et al., 1966) is of particular interest. This phospholipid is very similar in its charge characteristics to lipid A and the lipid A precursor and demonstrates almost identical affinity constants for both Ca^{2+} and Mg^{2+}. In addition, the fractional stoichiometries of binding to lipid A and precursor are close to those observed for phospholipid molecules chelating cations through their phosphate groups (Abramson et al., 1966; Hauser et al., 1976). It would seem reasonable that these phosphate groups in native lipopolysaccharide form the low-affinity site. In analogy with sialic acid (Jaques et al., 1977) and hyaluronic acid (Winter & Arnott, 1977) and the known ability of carboxylate clusters to form strong metal complexes, we propose that the highly negative KDO region (Figure 1) binds 1 mol of divalent cation per mol of lipopolysaccharide.

The observed interaction of polymyxin B with DNS–lipopolysaccharides confirms the existence of high-affinity binding site(s) for this ligand (Storm et al., 1977), but the site(s) of binding has not been specifically identified. The K_d values obtained for binding to DNS–lipopolysaccharides (0.3–0.5 μM) were somewhat lower than those reported for interaction with bacterial phospholipids (2.5–5 μM) (Storm et al., 1977). It is possible that the antibiotic binds relatively nonselectively to both the KDO and phosphate groups of lipopolysaccharide.

The potential physiological functions of the high-affinity binding of divalent cations remain to be established. There is some evidence that the KDO region is important for the translocation and integration of lipopolysaccharide into the outer membrane. Thus, the KDO-deficient lipid A precursor formed by KDO mutants of *S. typhimurium* (Rick & Osborn, 1977) is poorly translocated to the outer membrane (Osborn, Rasmussen, and Rick, unpublished experiments). In addition, the KDO region could have a secondary role in the formation of the lipopolysaccharide-dependent barrier function of the outer membrane. Phosphate-mediated ionic bridges linking lipopolysaccharide or lipopolysaccharide–protein complexes in the outer membrane may be the major force in establishing the principal permeability barrier. However, the KDO site could provide another way of promoting lipopolysaccharide–lipopolysaccharide interaction by masking the anionic charges of the KDO molecules. In addition, this site could alter the interactions of the polysaccharide side chains with each other, increasing hydrogen bonding and resulting in a more rigid and impermeable membrane. A number of investigators have demonstrated a role of the polysaccharide side chains in the barrier function of outer membrane (Galanos et al., 1977; van Alpen et al., 1977; Nixdorff et al., 1978). By analogy, Ca^{2+} is known to interact with negatively charged sugars such as hyaluronic acid to form stiffened chain segments and extended hydrogen-bonded three-dimensional meshworks in connective tissue (Winter & Arnott, 1977).

References

Abramson, M. B., Katzman, R., Gregor, H., & Curci, R. (1966) *Biochemistry 5*, 2207–2213.

Ames, B. N. (1966) *Methods Enzymol. 8*, 115–118.

Bader, J., & Teuber, M. (1973) *Z. Naturforsch., Teil C 28*, 422–430.

Dröge, W., Lehmann, V., Lüderitz, O., & Westphal, O. (1970) *Eur. J. Biochem. 14*, 175–184.

Galanos, C., & Lüderitz, O. (1975) *Eur. J. Biochem. 54*, 603–610.

Galanos, C., Lüderitz, O., & Westphal O. (1969) *Eur. J. Biochem. 9*, 245–249.

Galanos, C., Lüderitz, O., Rietschel, E. T., & Westphal O. (1977) *Int. Rev. Biochem. 14*, 239–334.

Hauser, H., Darke, A., & Phillips, M. C. (1976) *Eur. J. Biochem. 62*, 335–344.

Jaques, L. W., Brown, E. B., Barrett, J. M., Brey, W. S., & Weltner, W., Jr. (1977) *J. Biol. Chem. 252*, 4533–4538.

Kinoshita, T., Iinuma, F., & Tsuji, A. (1974) *Anal. Biochem. 61*, 632–637.

Lehmann, V., Redmond, J., Egan, A., & Minner, I. (1978) *Eur. J. Biochem. 86*, 487–496.

Leive, L. (1974) *Ann. N.Y. Acad. Sci. 235*, 109–129.

Nixdorff, K., Gmeiner, J., & Martin, H. H. (1978) *Biochim. Biophys. Acta 510*, 87–98.

Osborn, M. J. (1963) *Proc. Natl. Acad. Sci. U.S.A. 50*, 499–506.

Osborn, M. J. (1969) *Annu. Rev. Biochem. 38*, 501–538.

Osborn, M. J., Rosen, S. M., Rothfield, L., & Horecker, B. L. (1962) *Proc. Natl. Acad. Sci. U.S.A. 48*, 1831–1838.

Osborn, M. J., Gander, J. E., Parisi, E., & Carson, J. (1972) *J. Biol. Chem. 247*, 3962–3972.

Rick, P. D., & Osborn, M. J. (1977) *J. Biol. Chem. 14*, 4895–4903.

Rick, P. D., Fung, L. W.-M., Ho, Ch., & Osborn, M. J. (1977) *J. Biol. Chem. 252*, 4904–4912.

Romeo, D., Girard, A., & Rothfield, L. (1970) *J. Mol. Biol. 53*, 475–490.

Sanderson, K. E., MacAlister, T. J., & Costerton, J. W. (1974) *Can. J. Microbiol. 20*, 1135–1145.

Scatchard, G. (1949) *Ann. N.Y. Acad. Sci., 51*, 660–672.

Schindler, M., Assaf, Y., Sharon, N., & Chipman, D. M. (1977) *Biochemistry 16*, 423–431.

Sillerud, L. O., Prestegard, J. H., Yu, R. K., Schafer, D. E., & Konigsberg, W. H. (1978) *Biochemistry 17*, 2619–2628.

Storm, D. R., Rosenthal, K. S., & Swanson, P. E. (1977) *Annu. Rev. Biochem. 46*, 723–763.

Strominger, J. L., Park, J. T., & Thompson, R. E. (1959) *J. Biol. Chem. 234*, 3263–3268.

van Alpen, L., Lugtenberg, B., van Boxtel, R., & Verhoef, K. (1977) *Biochim. Biophys. Acta 466*, 257–268.

van Alpen, L., Verkleij, A., Leunissen-Bijvelt, J., & Lugtenberg, B. (1978) *J. Bacteriol. 134*, 1089–1098.

Verkleij, A., van Alpen, L., Bijvelt, J., & Lugtenberg, B. (1977) *Biochim. Biophys. Acta 466*, 269–282.

Voss, J. G. (1967) *J. Gen. Microbiol. 48*, 391–400.

Weigele, M., De Bernardo, S., Tengi, J., & Leimgruber, W. (1972) *J. Am. Chem. Soc. 94*, 5927–5928.

Williams, R. J. P. (1972) *Physiol. Chem. Phys. 4*, 427–439.

Winter, W. T., & Arnott, S. (1977) *J. Mol. Biol. 117*, 761–784.